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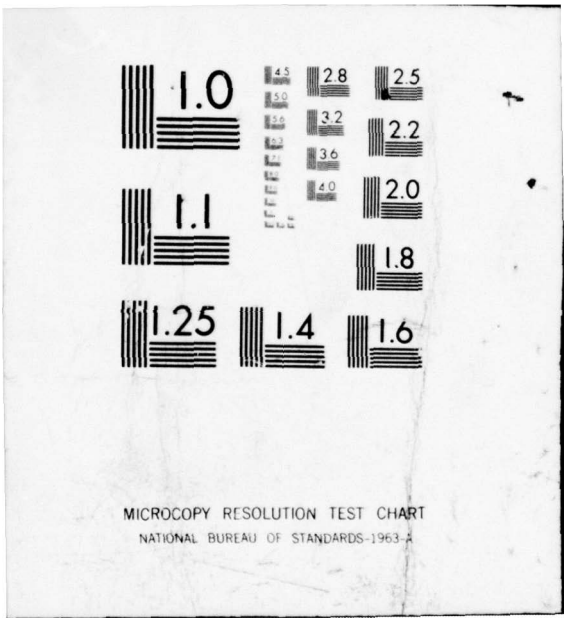
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PRESERVATION OF HUMAN GRANULOCYTES

II. CHARACTERISTICS OF GRANULOCYTES OBTAINED BY COUNTERFLOW CENTRIFUGATION

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PRESERVATION OF HUMAN GRANULOCYTES

II. CHARACTERISTICS OF GRANULOCYTES OBTAINED BY COUNTERFLOW CENTRIFUGATION

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Short Title: PROPERTIES OF ELUTRIATED HUMAN GRANULOCYTES

Counterflow centrifugation can isolate human granulocytes quantitatively from a 10 ml volume of whole blood or buffy coat. The isolated granulocytes are free of lymphocytes, erythrocytes and platelets. Leukocytes isolated by sedimentation procedures usually have ten times more erythrocytes than granulocytes and significant numbers of lymphocytes and platelets.[1-6] Granulocytes isolated by continuous-flow centrifugation and nylon filtration are also contaminated with significant numbers of erythrocytes, platelets, and lymphocytes.[7-9]

In this study granulocytes isolated by counterflow centrifugation in the Beckman JE-6 elutriator rotor were compared with those obtained by dextran or physiogel sedimentation or by adhesion filtration using nylon fibers.

MATERIALS AND METHODS

Fifty ml of blood was collected by the American Red Cross from healthy donors in specially prepared plastic bottles containing acid-citrate-dextrose (ACD, NIH, Formula A), citrate-phosphate-dextrose (CPD), or heparin anticoagulants. Within two hours after collection, the blood was separated by centrifugation in a Beckman JE-6 elutriator rotor held in a J-21B centrifuge (Figure 1). The rotor was designed to separate granulocytes in an isotonic medium in a 4.5 ml chamber opposed by a counter-balanced bypass chamber in a seal assembly. A strobe light permitted visualization of the cells in the chamber during separation. A digital tachometer measured the rotor speed. The rates of flow of cell suspensions passing through the rotor were controlled by a Cole-Palmer #7545 Masterflex pump with an external potentiometer to control the flow rates to within 0.2 ml per minute between 10 to 30 ml per minute.

FIG. 1

Separation Procedure

The harness (Figure 1) was flushed with isotonic phosphate buffered sodium chloride solution containing 0.6% human albumin (280 mOsM). The rotor was filled by leaning the head on its side with the separation chamber perpendicular to the bench and the buffer was forced into the inflow port from a syringe, taking care to eliminate air bubbles. The head was placed in the centrifuge, the harness attached, the temperature set at 10°C, and the speed at 2,000 to 2,080 rpm. A 10 ml sample of whole blood or buffy coat was introduced into the mixing chamber in the vertical position by means of a three-way stop cock. Once in the chamber, the sample was mixed with buffer at an 11.8 ml/min. flow rate. This was increased to 12.6 ml/min. after 10 minutes; to 13.2 ml/min. after 15 minutes, and then to 14.0 ml/min. for the remainder of the procedure, which required 35 to 40 minutes and used 400 to 450 ml of buffer. The head was then dismantled, the separation chamber removed, and the

granulocyte concentrate aspirated using a blunted #18 guage needle attached to a 10 ml syringe.

Testing Procedures

Cell counts were obtained in a Coulter Model F counter and by microscopic enumeration using a hemocytometer. A Coulter Channelyzer Model ZH was used for size distribution analysis. Red cell contamination was determined by integrating counts for the red cell and granulocyte peaks and calculating the red cell fraction of the total count. For electronic counting or sizing, concentrations of the separated granulocytes of 5.0×10^6 per ml were diluted with a 0.02 M phosphate buffer in isotonic sodium chloride containing human albumin. Size distributions were studied in 3.0×10^6 cells suspended in 20 ml of medium to a final concentration of 150,000 per ml. Before sizing, cell-free medium at the same temperature was flushed through the aperture of the counter.

Recovery of the isolated granulocytes was determined from

the total available granulocytes in whole blood (white blood cells per ml X volume of whole blood X % PMN's from Wright's stained smears) and the total granulocytes recovered in the elutriated sample (white blood cells per ml X volume in chamber X % PMN's from Wright's stained smears). The RBC:WBC ratio was obtained by integrating the red cell peak and granulocyte peak on the Coulter tracing and by microscopic enumeration of the cells in a hemocytometer. The purity of the white blood cell population was determined from white blood cell differential counts made of stained smears of elutriated cell suspensions.

Morphology of granulocytes was studied with scanning electron microscopy, light microscopy, and fluorescence microscopy. For the electron microscope, 1 ml of granulocytes (ca. 6.0×10^6 per ml medium) were fixed in 9 ml of Millonig's buffer containing 2% gluteraldehyde.[10] After the cells were washed and coated, they were examined with a scanning electron microscope (U3-JEOL, Japan).

Fluorescence studies were carried out with an Olympus Vanox transmission fluorescence microscope with an illumination unit A-FL containing excitor barrier, and heat absorbing filters. The power supply unit (115 AC input, DC output) fired a super pressure Mercury burner type L-2. Cells in wet mounted thin preparations were viewed ordinarily for identification with green excitor filter (G5330) and viewed for fluorescence with UV excitor filter (Schott BG-12) and blue barrier filter (Schott OG-530).

Membrane integrity was estimated indirectly from esterase activity in cytoplasm of granulocytes determined as the percentage of cells exhibiting green fluorescence after a 5-minute incubation with fluorescein diacetate (FDA) according to the method of Dankberg and Persidsky.[11] It is based on studies of fluorescence in cytoplasm of fluorescein from esterases acting on fluorescein diacetate as described by Rotman and Papermaster[12] and on studies of nuclear fluorescence

with ethidium bromide as described by Edidin.[13] Simultaneously, nuclear membrane integrity was estimated as the percent of cell nuclei staining with ethidium bromide. Solutions were made of fluorescein diacetate (FDA, 5 mg per ml in acetone kept at -20°C) and ethidium bromide (200 mg per ml in acetone kept at -20°C) and ethidium bromide (200 ug per ml in Hank's solution, HBSS-Ca^{++}-Mg^{++}). From these a working solution was prepared by mixing 1 ml of ethidium solution, 50 ul of FDA solution, and 49 ml of HBSS-Ca^{++}-Mg^{++}.

The test was performed by incubation of 0.5 ml of the FDA-ethidium mixture with 0.3 ml of granulocyte suspension containing 1 to 4 million granulocytes for 5 minutes at room temperature. Thin wet mounts were made, tamped to reduce thickness, and viewed. Granulocytes were identified first with the excitor filter (G5330). The same fields were viewed again after changing to excitor filter (Schott BG-12) and barrier filter (Schott OG-515 or 530). These were counted as green

cytoplasmic fluorescent cells or as ethidium reactive red fluorescent nuclei. Two hundred cells were counted.

Phagocytosis was measured by fluorescence microscopy with latex particles conjugated with a fluorescent chromophore (Fluolite DS-5005 was obtained from ICI, Finland) according to the procedure of Arvilommi.[14] The particles were previously incubated with fetal calf serum and subsequently washed twice with Hank's solution (HBSS). One ml of granulocytes (ca. 6×10^6) was incubated with 0.1 ml particles (30×10^6) for 10 minutes at 37°C. HBSS (5 ml) was added at 22°C and the suspension centrifuged (225 X g) for 2 minutes. The supernatant was aspirated and 0.5 ml of the FDA-ethidium bromide mixture added. The suspension was mixed and examined after 5 minutes at 22°C. The cells were viewed exactly as in the FDA-ethidium test and a phagocytic index determined as the percent containing four or more fluorescent particles. The capacity of granulocytes to ingest large numbers of particles was also estimated as the

percentage containing 30 or more particles.

Oxygen consumption by granulocytes was measured polarographically by the method described by Crowley, Skrabut and Valeri[15] using an oxygen probe marketed by the Yellow Springs Company.

Nucleotides were determined by a modified procedure described by Scholar et al.[16] High pressure anion exchange chromatography on 1 cm X 25 cm columns of Partisil-10-SAX with a Varian liquid chromatograph at ambient temperature was used. Granulocyte suspensions containing less than 5 percent red cells (ca. 20 X 10^6 cells in 3 ml) were centrifuged at 3,000 X g for 3 minutes, the supernatant removed, and 3 ml of ice cold trichloroacetic acid added to the pellet. The extract was mixed, stored in ice for 1 hour and centrifuged at 12,000 X g for 20 minutes. The extract was transferred to a 50 ml glass stoppered pyrex round bottom glass tube and extracted eight to ten times with water saturated diethylether (10 volumes ether

to 1 volume extract) at 4°C. The aqueous extract was frozen in a dry ice-alcohol bath, the excess ether decanted, and the tubes lyophilized. The dry residue was dissolved in 0.1 ml of water and 25 ul aliquots were chromatographed. The full scale absorbency for these samples was either 0.04 or 0.08 OD units. A linear gradient was used in which the low concentration eluent was 0.015 M KH_2PO_4 and the high concentration was 0.25 M KH_2PO_4 in 2.2 M KCl. Identification and quantitation of nucleotides were the same as described.[16]

Chemotaxis was measured with a modified Boyden technique according to the procedure of Smith et al.[17] One ml of sedimented or elutriated granulocytes diluted to 1 X 10^6 was added to the chambers, the lower portion filled with lipopolysaccharide endotoxin (LPS) in HBSS (10 ug per ml HBSS). The LPS was previously incubated for 30 minutes at 37°C in HBSS containing 10% fresh autologous plasma. The cells were separated by a 3 um Millipore filter from the endotoxin. The chambers were incubated for three hours at 37°C in a 95%

air-5% CO_2 humidified atmosphere. After incubation, the filters were stained with hematoxylin and eosin and the cells which had traversed the filters counted in ten high power fields. Granulocytes in the plane of the filter surface were not counted.

RESULTS AND DISCUSSION

The results of 59 studies performed on a 10 ml volume of whole blood collected in different anticoagulant solutions are reported in Table 1. From all of these, 91% of the granulocytes available in the whole blood were isolated. Examinations of films stained with Wright's stain showed them to be 97% polymorphonuclear cells. An occasional large lymphocyte was observed and the other cells comprised of leukocytes in a ratio of two eosinophils to one basophil. When the time in the rotor was shortened to 25 to 30 minutes, 3% lymphocytes and 2% monocytes accompanied granulocytes in the final suspension. Red cell contamination was small and variable, and was found to depend on the concentration of anticoagulant; the ratio of PMNG to red cells was 4.6 to 1 in 25% ACD and 11 to 1 in 7.5% ACD. Whole blood collected into heparin gave results which were similar to those observed when blood was collected in 7.5% ACD. The best results were obtained by isolating

TABLE 1

granulocytes in a 10 ml volume of buffy coat obtained from whole blood collected in CPD. The four experiments reported in Table 1 show a recovery of 97% of white blood cells present, a purity of 96% of granulocytes, and a red cell contamination of 2.5%.

In our previous studies isolating granulocytes from the buffy coat of blood by sedimentation with 4% hydroxyethyl starch, the isolated granulocytes had a PMNG to lymphocyte ratio of 5.7 to 1 and a red cell to leukocyte ratio of 9 to 1.[6]

The purity of the granulocytes isolated by counterflow centrifugation was shown by volume distribution analysis. When granulocytes were isolated from the whole blood of one donor by sedimentation with dextran and by counterflow centrifugation with the JE-6 rotor, one well-defined population of PMNG was observed in the granulocytes isolated by elutriation. (Figure 2). The median channel #56 corresponded to the median volume in the granulocyte population, and a small

FIG. 2

population of red cells at median channel #10 was evident. The symmetry and sharpness of the granulocyte peak was consistent with a homogeneous distribution of granulocytes whose volumes varied over a narrow range. The granulocytes isolated by dextran sedimentation showed the presence of three cell types: red cells (channel #10), lymphocytes (channel #33), and granulocytes (channel #58). The median channel of granulocytes isolated by dextran sedimentation of blood demonstrated a wider distribution of volumes than did granulocytes isolated by elutriation. Red cells have been identified by the study of volume distribution of cells in buffy coat obtained by sedimentation of red blood cells with HES[18] and in murine mouse marrow cells.[19] Electronic display of the size distribution of cells on an oscilloscope provided a rapid assessment of the quality and purity of granulocytes isolated by elutriation.

Granulocytes isolated by counterflow centrifugation had green fluorescence when tested with fluorescein diacetate.

indicating that cytoplasmic esterase activity was present. One to two percent of the cells also reacted with ethidium bromide. These results indicated that all but one to two percent of the granulocytes had intact nuclear membranes which prevented ethidium from interacting with nucleic acids of the cell nuclei.

Ethidium exclusion by granulocyte nuclei was compared to that of trypan blue. In two experiments trypan blue positive cells and ethidium uptake paralleled each other through the various manipulations of the granulocytes (Table 2). Granulocytes collected optimally using counterflow centrifugation excluded ethidium bromide and trypan blue and all showed active cytoplasmic esterases (Table 2). After freezing in DMSO and thawing, about 25 percent of the cells were unable to exclude trypan blue and ethidium bromide. Washing further reduced the number of dye exclusive cells, and incubation after the freeze-thaw-wash procedure for 2 hours at 37°C indicated that 75 to 90 percent could not exclude the dyes. The presence

TABLE 2

of 1.25% albumin in experiment 1 compared to 0.61% albumin in experiment 2 may account for the improved granulocyte stability due to a protective effect on the membrane.

Examination of granulocytes prepared by counterflow centrifugation with the elutriator rotor revealed morphological and surface properties similar to granulocytes obtained from buffy coats of heparinized whole blood.[20] The granulocytes were rounded with irregular surfaces and there was much exoplasm consisting of folded membranes and surface convolutions. Leafy or veil-like projections of the exoplasm extended into the medium (Figure 3A). Polliack et al.[20] have reported that granulocytes have microvilli, ridge-like profiles and ruffled membranes. Granulocytes collected by adsorption on columns of nylon fibers showed some marked alteration of structure (Figure 3B). These granulocytes were spherical, with surface convolutions but no ruffles or leafy veils. The filimentous material seen in the background

FIG. 3A

FIG. 3B

was the matrix on which the cells were fixed. Removal from nylon produced granulocytes devoid of microvilli and folded membranes, a process which may be due entirely to our method of adsorption and collection of granulocytes from nylon fibers. In other studies, thymocytes removed from nylon fiber had microvilli present on cell surfaces.[21]

The capacity for metabolism of elutriated granulocytes was evaluated by measurements of adenine, guanine, di and trinucleotide levels and the hexosemonophosphate shunt activity measured polarographically as oxygen consumption with latex stimulated cells (Table 3). These biochemical measurements for elutriated granulocytes are in agreement with the values reported by Scholar et al.[16] for lymphocytes and granulocytes. The ATP to ADP ratio of 10.4 is similar to that of 9.9 for lymphocytes and granulocytes isolated by sedimentation with Plasmagel and 8.3 for lymphocytes and granulocytes isolated by adsorption reversal from cotton wool.[16] These biochemical

TABLE 3

measurements indicate that little damage occurred during collection of granulocytes by the elutriation procedure. Subsequent studies yet to be reported have shown that the nucleotide levels were greatly diminished in granulocytes following liquid storage, freezing, and washing.

Oxygen uptake of elutriated granulocytes incubated with latex had a normal response to stimulation (Table 3). The initial rate was not significantly different from that in cells sedimented with dextran or physiogel (Table 3). Stimulation with latex produced a response similar to that in granulocytes isolated by sedimentation of red blood cells with dextran or physiogel.

Chemotactic and phagocytic activity was similar for both the HES-sedimented and the elutriated granulocytes (Table 3). In two experiments on chemotaxis, the controls were similar. A higher ratio of endotoxin stimulation for elutriated cells in one experiment is unexplained.

The ultimate goal of this work is to preserve human

granulocytes by freezing. Granulocytes isolated by centrifugal elutriation are homogeneous, and the absence of lymphocytes and red blood cells allows for study of granulocytes only. Many reports of white cell preservation in the past actually dealt with red cell suspensions enriched with white blood cells. The effects of preservation by liquid and freeze technology on homogeneous granulocytes can now be studied. Granulocyte volume measured by electronic sizing and fluorescence tests of membrane integrity are <u>in vitro</u> viability characteristics which can be used to evaluate the efficacy of preservation.

ACKNOWLEDGMENTS

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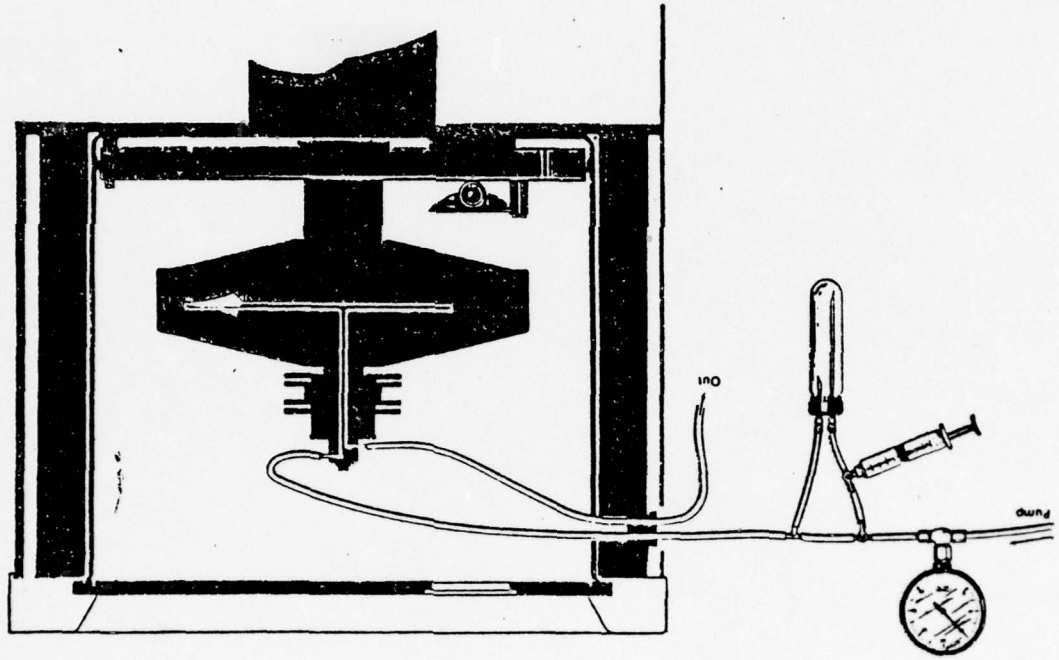
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FIGURE 2

Size distributions of leukocytes obtained by a dextran sedimentation procedure and counterflow centrifugation. Granulocytes in the top curve were obtained by sedimentation of whole blood with dextran as described in Reference 5. Granulocytes in the bottom curve were obtained as described in this paper. Granulocytes were suspended in 10 ml of phosphate buffered saline (PBS) albumin, pH 7.1, with a count of 200,000 per ml and 0.1 ml volume sized in the Coulter Channelyzer Model ZH.

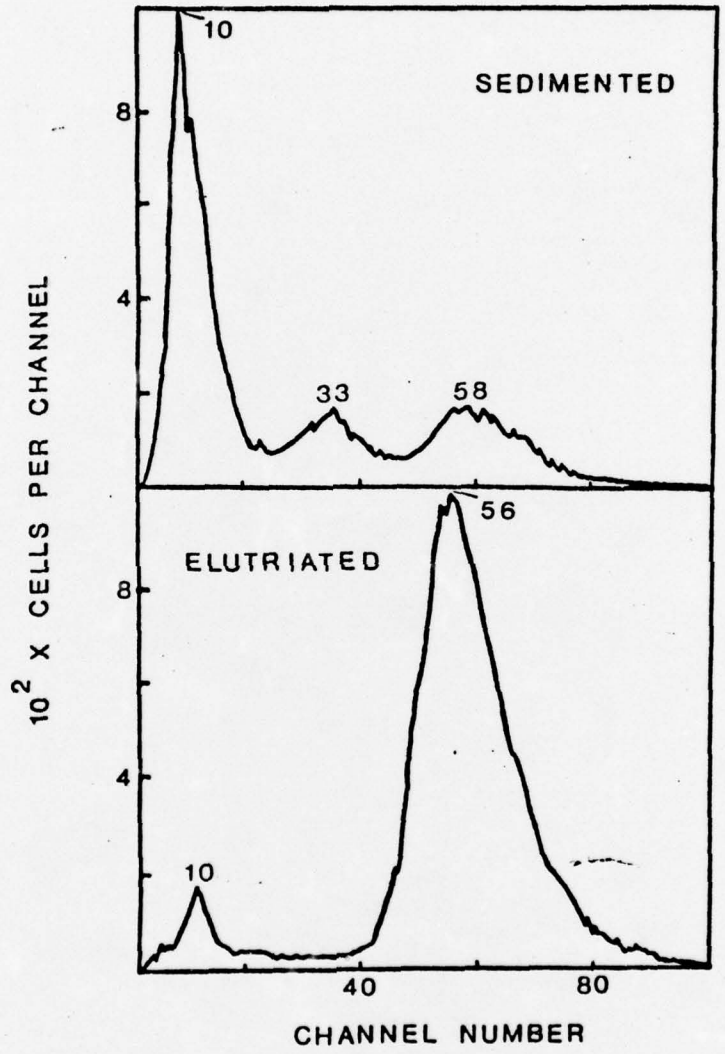

FIG 2 F.J.C. et al

FIGURE 3

Scanning electron photographs of granulocytes obtained by elutriation (Fig. 3A) and by adsorption and release from nylon fiber (Fig. 3B). Cells were fixed 1:10 in 2% gluteraldehyde in Millonig's buffer. Magnification is 3,000 X.

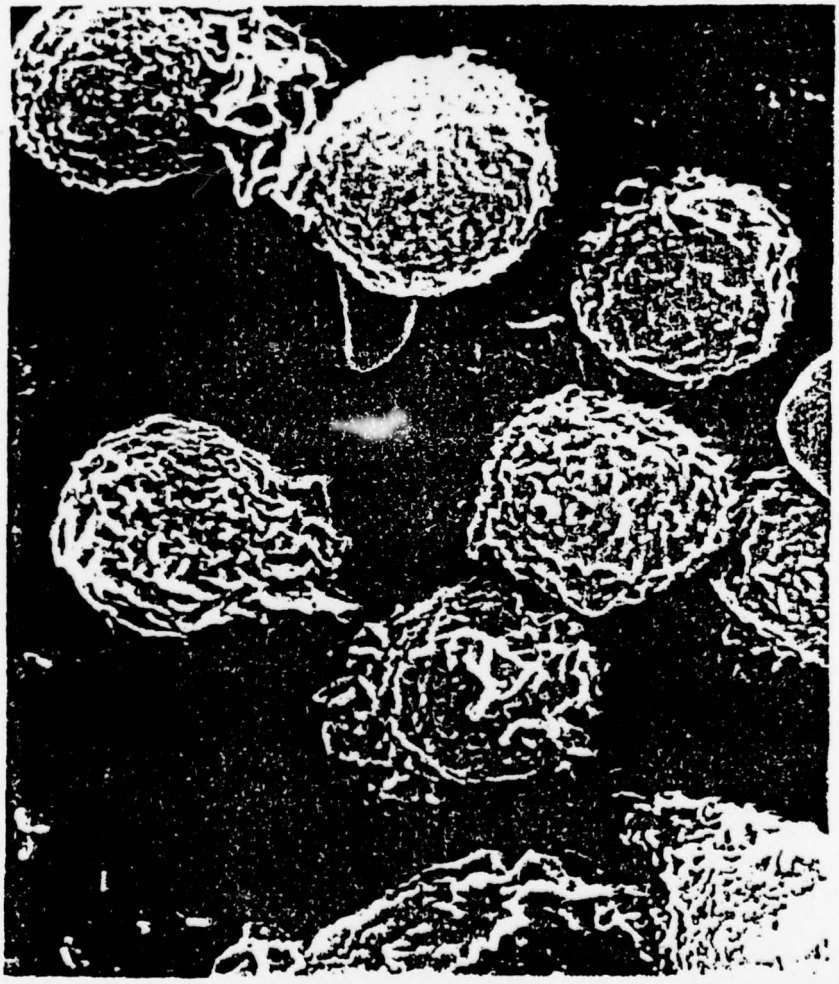

FIG. 3A F.J. et al

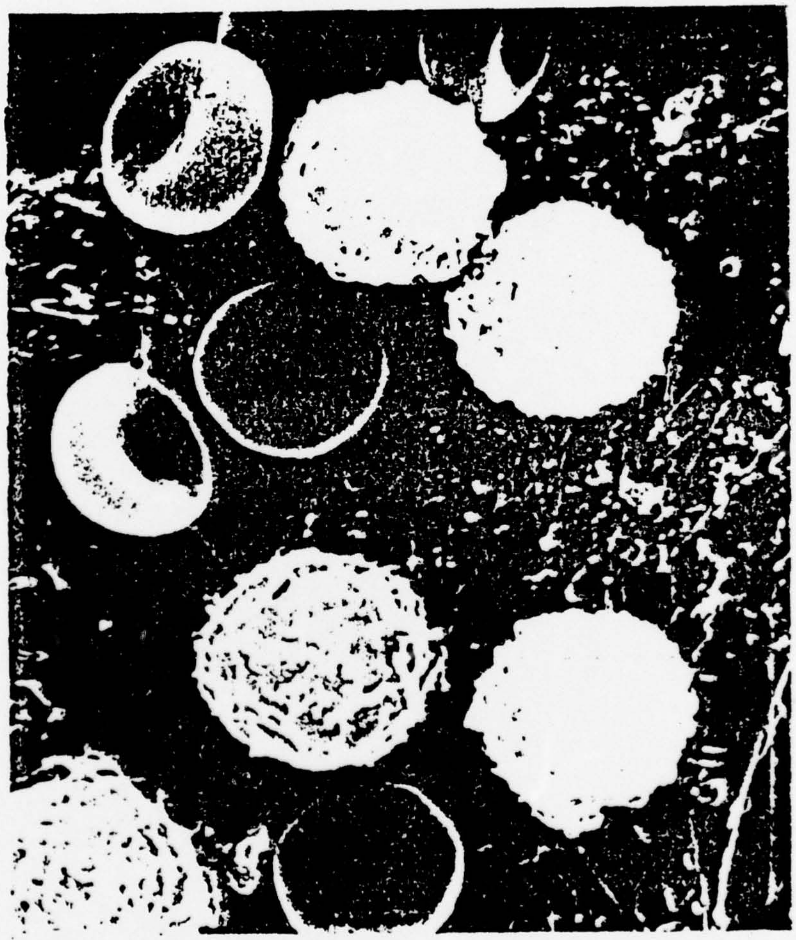

FIG. 3B [illegible]

TABLE 1

Isolation of Granulocytes from Whole Blood and Buffy Coat By Counterflow Centrifugation
Blood Collected in ACD, CPD, or Heparin

Number of Experiments	Anticoagulant	Recovery (% of PMNG)	WC Differential Count (%)			PMNG	Fluorescence (%)	
			Polys	Lymphs	Other	RBC	Green	Red
6	Heparin	80 ± 15	99.0 ± 0.6	0.5 ± 0.6	0.8 ± 0.8	10	98.6 ± 1.5	1.0 ± 0.0
	ACD (% v/v)*							
21	25	92 ± 12	97.4 ± 2.1	0.8 ± 0.7	1.8 ± 2.2	4.6	98.6 ± 1.3	1.5 ± 1.3
14	22	91 ± 11	96.4 ± 2.1	1.4 ± 1.1	2.2 ± 1.5	6.9	98.4 ± 0.7	1.6 ± 0.7
3	15	88 ± 7	96.9 ± 1.7	1.8 ± 1.9	1.7 ± 0.3	7.2	98.7 ± 0.3	1.3 ± 0.3
9	7.5	90 ± 8	96.8 ± 2.6	1.2 ± 1.2	1.8 ± 1.5	11	98.2 ± 0.5	2.0 ± 0.7
6	CPD	99 ± 3	98.8 ± 0.4	0.5 ± 0.3	0.7 ± 0.3	24	98.3 ± 0.5	1.7 ± 0.5
59	Overall	91 ± 11	97.3 ± 2.1	1.0 ± 0.9	1.7 ± 1.7	8.8	98.5 ± 0.9	1.5 ± 0.9
6	Buffy Coat (ACD)	99 ± 3	95.3 ± 2.9	3.2 1.8	1.3 ± 1.0	17	98.5 ± 0.6	1.5 ± 0.6
4	" " (CPD)	97 ± 4	96.0 ± 2.0	2.3 1.9	1.8 0.5	41	98.5 ± 0.6	1.5 ± 0.6

*ACD, 15% = normal volume concentration: values are means ± S.d.

TABLE 2

Uptake of Dyes by Granulocytes Isolated by Elutriation and then Preserved

Condition	Experiment	Uptake (% of cells)		
		Trypan Blue	Ethidium	Fluorescein Diacetate
After Isolation	1	0	1	99
	2	3	2	98
Frozen with 5% DMSO at 2°C per minute and stored at -80°C for 3 days and thawed at 37°C (44°/min)	1	23	24	76
	2	25	21	79
Frozen-thawed-washed granulocytes	1	22	22	78
	2	37	40	60
Frozen-thawed washed granulocytes incubated at 37°C				
Hours				
1	1	39	40	60
	2	75	72	28
2	1	79	75	25
	2	80	91	9

In experiment 1 and 2, 1.25 and 0.61% human albumin, respectively, were present.

Two ml of granulocytes (6 2 X 10^6) in PBS with albumin were frozen. After thawing they were diluted with an equal volume of HBSS and centrifuged at 75 X g for 8 minutes at 4°C. They were resuspended in 2.0 ml of HBSS and incubated for 2 hours at 37°C.

roperties of Granulocytes Isolated By Counterflow Centrifugation (Elutriation) and By Sedimentation With Physiogel, Dextran, and Hydroxyethyl Starch (HES)

Nucleotide Content of Granulocytes Isolated By Elutriation (nMoles/10^6 cells)

ADP	ATP	GPD	GTP	ATP/ADP
0.11	1.13	0.02	0.38	10.3

Oxygen Uptake (ul $O_2/10^7$ cells/hour

Number (n)	PMNG Obtained With	Basal Rate	Stimulated Rate
24	Dextran	14.7 ± 5.2	103 ± 12
24	Physiogel	14.7 ± 5.9	92 ± 13
6	Elutriation	13.0 ± 9.1	107 ± 16

Chemotaxis (# cells/10 HP fields/10^6 cells incubated

	Expt.	Control	Endotoxin	Endo/Control
ES-Sedimented	1	44	92	2.1
ells	2	50	129	2.6
lutriated	1	40	120	3.0
ells	2	30	320	10.7

Phagocytosis (%)

	Expt.	Index	Capacity
ES-Sedimented	1	86	39
ells	2	77	15
lutriated	3	73	24
ells	4	84	70
	5	77	38

Index is the percent of cells containing 4 or more particles

Capacity is the percent of cells containing 30 or more particles

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Human granulocytes were isolated from whole blood and from the buffy coat of blood by counterflow centrifugation using the Beckman JE-6 rotor. Ninety-one percent of granulocytes in the human blood sample were isolated in 59 studies. The differential white blood cell count showed 97% polys, 1% lymphs, and 2% others. The ratio of polymorphonuclear white blood cells to red blood cells was 9:1 when the granulocytes were isolated from whole blood and 40:1 when the granulocytes were isolated from the buffy coat of CPD-collected blood.

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The size distribution showed a single symmetrical peak. Measurements of oxygen consumption, chemotaxis, and phagocytosis in granulocytes isolated by counterflow centrifugation were similar to those in granulocytes isolated from blood by dextran sedimentation. The granulocytes had normal adenine and guanine di- and trinucleotide levels.

Scanning electron microscopy demonstrated spherical granulocytes with ridge-like profiles and leafy folded exoplasm. After fluorescein diacetate treatment, the cytoplasm of 98.5 ± 0.7% of fresh granulocytes was positive, while 98.5 ± 1.2% of the fresh granulocytes excluded ethidium bromide from their nuclei. The response of freeze-preserved granulocytes to treatment with fluorescein diacetate and ethidium bromide showed that 75% of granulocytes survived freezing and thawing in 5% DMSO but were progressively unstable after incubation for 2 hours at 37°C.